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**Is there an ideal protocol for sampling macroinvertebrates in springs?**


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**Abstract**

Sampling in springs has several technical problems due to their reduced dimensions and habitat heterogeneity. A standardised quantitative method for sampling crenic macroinvertebrate has never been proposed. The aim of this study is to compare different sampling methods and considering their environmental impacts. Firstly, we present a review of sampling methods found in the literature and discuss their advantages and disadvantages with respect to selective gathering of the target community and habitat disturbance. Altogether, ten different methods have been reported, the use of nets being the most common protocol. Secondly, we report the results of macroinvertebrate samplings performed in three springs, each surveyed twice, using three different methods (multi-habitat proportional hand net, baited traps and vegetation washing), in order to compare their effectiveness in collecting macroinvertebrates. Overall 32 macroinvertebrate taxa, mostly identified at family level, were collected in the sampled springs. Significant differences in abundances were found using different methods, while results on community structure were comparable between the hand net sampling and the combined use of the other two methods, although with slight differences in the composition of Coleoptera and Diptera assemblages. The hand net, with a multi-habitat proportional approach, provided more thorough results, making it suitable for biodiversity inventories but having some potentially negative effects on spring habitats. Traps and vegetation washing are also reliable methods with negligible impacts on spring ecosystems, that can be conveniently used in ecological studies.

**Keywords**: springs, sampling methods, macroinvertebrates, biodiversity, impact assessment.

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## Introduction

In spite of the their small size, the spring 'mosaic' ecotonal structure results in a number of microhabitats that sustain high species richness (Cantonati *et al.* 2012). Several studies have highlighted the high biodiversity of macroinvertebrates in springs and the presence of rare and endemic species (e.g. Takhteev *et al.* 2010; Maiolini *et al.* 2011; Kubíková *et al.* 2012; Martin & Brunke 2012; Spitale 2012; Spitale *et al.* 2012). Despite great interest in spring biodiversity, a standardised quantitative method for sampling crenic macroinvertebrate taxa has never been developed. On the contrary, the use and limits of various standard methods for sampling aquatic benthic macroinvertebrates have been extensively discussed (e.g. Davies 2001). The technical difficulties of sampling in springs were well summarised by Gerecke and co-authors (2007): 'The main dilemma of limnological studies in springs probably derives from the generally reduced dimensions and extreme heterogeneity of the habitat'. Furthermore, many authors (Gerecke *et al.* 1998; Zollhöfer 1999; Myers & Resh 2002; Staudacher & Füreder 2007; Tichá *et al.* 2012) noted that some surveys, which involved samplings in all microhabitats, could be destructive for the environment and the biota of these fragile ecosystems. Cantonati and colleagues (2007) suggested effective methods for collecting spring invertebrates, but a variety of methods have been adopted in crenic investigations. Previous studies on macroinvertebrates in different aquatic ecosystem have shown that sampling methods affect the data precision, and the selection of sampling technique is among the most important decisions for freshwater studies (Carter & Resh 1993). Standardise the sampling procedure is thus necessary in order to obtain precise and comparable biological data for spring surveys and assessment.

The aim of this study was to summarize sampling methods in springs and to compare the effectiveness of some semi-quantitative sampling methods, taking into account their potential impacts on spring habitat and biota.

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Standard Surber samplers (sampling area: 0.09-0.1 $m^2$) have been rarely used (Smith et al 2003; Barquín & Death 2008). More frequently, smaller samplers were preferred (Erman & Erman 1995; Erman 1998; Zollhöfer 1999; von Fumetti *et al.* 2006; Gerecke *et al.* 2011). Mesh size of Surber, kick or hand nets varies from 100 µm to 1 mm. Gerecke *et al.* (2007) suggested to consider different microhabitats when sampling in springs, if possible respecting the relative microhabitat covering. Few studies chose a multi-habitat proportional approach (Crema *et al.* 1996; Zollhöfer 1999; Martin & Brunke 2012) or tried to sample all the substrates (Bonettini & Cantonati 1998; Mezzanotte & Sambugar 2004; Ilmonen *et al.* 2012).¶
Moreover, many authors (Gerecke *et al.* 1998; Zollhöfer 1999; Myers & Resh 2002; Staudacher & Füreder 2007; Tichá *et al.* 2012) pointed out that a survey with net in all microhabitats could have serious consequences for the environment and the biota.

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## Materials and methods

Three rheocrenic, permanent springs located between 474 and 589 m a.s.l. in the 'Mount Prinzera' protected area (lat: 44°37'N; long. 10°03'E), an ophiolitic outcrop in northern Italy, near Parma, were selected for the study. Samplings were carried out in two seasons (May-June and August-September 2014). Macroinvertebrates were collected using three methods:

1- Multi-habitat proportional net: a hand net (frame dimensions: 10x10 cm; mesh size: 255 µm) was used for 10 replicas in each site. Substrate was sampled for an area equal to the net frame for 15 second for each replica. Every microhabitat was sampled for a number of replicas proportional to its percentage cover in the spring. For example given the substrate composition of 50% of gravel, 30% of mosses and 20% of silt, 5 replicas were done for gravel, 3 for mosses and 2 for silt. All the 10 replicas were composited into a single sample.

2- Vegetation washing: about 250 ml volume of submerged vegetation was collected and washed in laboratory through a 255 µm sieve.

3- Traps: following Bottazzi et al. (2011), these traps were derived from PASCALIS research project (Malard et al. 2002). They were built from PVC centrifuge tubes (length 100 mm; diameter 28 mm), by cutting the conical end, drilling an opening (0.5 cm of diameter) in its apex, and inserting it, inverted, into one end of the tube. The other end of the tube was closed with a 50 µm net. These traps were filled with washed and sieved gravel (0.3-1.0 cm). Traps were baited with corned meat, placed at the sediment-water interface, and covered with stones to keep them in place for 7-8 days. Two pairs of traps were deployed in each spring: one pair at the source, and the other 2 m downstream. For each pair, one trap was placed with the opening in the flow direction and the other in the opposite direction.

Vegetation washing and macroinvertebrate trapping were performed two weeks after the sampling.

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In the laboratory, collected material were washed through a 255 µm sieve and fixed with 90% ethanol. Macroinvertebrates were identified with Plecoptera, Trichoptera, Ephemeroptera, Coleoptera, Diptera, Crustacea to the family and Hirudinea, Gastropoda, Collembola, Hydrachnidiae, Odonata, Oligochaeta to coarser taxonomic level.

Differences in organism abundance between the three methods were tested with an Analysis of the Variance (ANOVA). Logarithmic transformation was used to obtain normal distribution and homogeneity of data, as determined by Shapiro and Bartlett tests (Legendre & Legendre 2012). Non-metric multidimensional scaling (NMDS, Legendre & Legendre 2012) was performed to evaluate possible differences in community structures determined by different methods. Centroids of methods were fitted on NMDS plots in order to identify these differences, then tested with Permanova (Anderson & Walsh 2013). Differences between methods were assessed by considering both the three different methods (net, vegetation washing, and traps) and combining data from vegetation washing and traps. Differences were also tested for each of the most diverse insect orders (Trichoptera, Coleoptera, and Diptera). Statistical analyses were performed using the R software, version 3.0.0 (R Development Core Team 2013), and vegan package version 2.0-7 (Oksanen et al. 2013).

**Results**

Ten different methods have been found; the use of hand or kick net is by far the most used protocol (table 1).

Overall 32 taxa were collected in our survey (Table 2). Insect orders with the highest number of families were Diptera (9), Trichoptera (7) and Coleoptera (6). Chironomidae was the most abundant taxon collected with the net (1029 specimens) and vegetation washing (60), whereas traps collected the highest number of Niphargidae (293). Lepidostomatidae, Chironomidae, Ceratopogonidae, Hirudinea, and Gastropoda were found in all samples collected by the net.

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The maximum number of taxa collected in one sampling session was eight using the net, and 11 combining traps and vegetation washing. Thirteen taxa were collected by all methods; net and the traps samplings shared seven taxa, whereas net and vegetation washing shared eight taxa. Finally, Hydropsychidae, Limnephilidae, and Hydrophilidae were only found in net samples, and Empididae were exclusively collected with traps (table 2).

Differences in taxa abundance between methods resulted significant, both considering the three distinct methods (fig. 1; $F = 16.180$; $p < 0.001$), and merging the traps and the vegetation washing (fig. 2; $F = 9.464$; $p = 0.012$).

Sampled communities formed three distinct groups near their centroids in the NMDS plot (stress = 0.15 – fig.3). This indicates differences in macroinvertebrate assemblages according to the methods, as confirmed by the Permanova test ($R^2 = 0.223$; $p = 0.010$). Stress was 0.13 in the plot of NMDS ordination obtained merging data collected with traps and vegetation washing (fig.4). The groups of the two different methods (net and traps plus washing vegetation) were less detectable. Permanova test ($R^2 = 0.136$; $p = 0.134$) indicated that there was not a significant difference between communities sampled with these two methods.

Net sampling and combined traps and vegetation samples showed differences for Coleoptera ($R^2 = 0.219$; $p = 0.030$) and Diptera ($R^2 = 0.250$; $p = 0.005$), but not for Trichoptera ($R^2 = 0.056$; $p = 0.826$) (fig. 5).

**Discussion**

The lack of a standardised sampling protocol for springs has led to the use of a wide variety of methodologies. Standard Surber samplers (sampling area: 0.09-0.1m$^2$) have been rarely used (Smith et al 2003; Barquín & Death 2008). More frequently, smaller samplers were preferred (Erman & Erman 1995; Erman 1998; Zollhöfer 1999; von Fumetti *et al.* 2006; Gerecke *et al.* 2011). The mesh size of Surber, kick or hand nets varies from 100 µm to 1 mm. Gerecke *et al.*

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(2007) recommended sampling different microhabitats in springs, at their relative microhabitat proportion. However, few studies have used a proportional multi-habitat approach (Crema *et al.* 1996; Zollhöfer 1999; Martin & Brunke 2012) or sampled all available substrates (Bonettini & Cantonati 1998; Mezzanotte & Sambugar 2004; Ilmonen *et al.* 2012). In addition, combined methods have been frequently used in the same study (Williams 1991; Erman & Erman 1995; Crema et al. 1996; Bonettini & Cantonati 1998; Erman 1998; Myers & Resh 2002; Sambugar *et al.* 2006; Staudacher & Füreder 2007; Bottazzi *et al.* 2011; Gerecke *et al.* 2011, Spitale 2012).

Each method has advantages and disadvantages that may be dependent on the specific aims of the study. Methods such as the use of sweep nets or emergence traps sample only organisms with aerial imagoes, whereas drift tubes/nets underestimate taxa not exposed to drift for behavioural or niche characteristics. Also methods that require collection by sight could be biased against small, more-mobile and less-visible organisms. Surber net, Bou-Ruch pump, and core-sampler may allow the collection of quantitative data, but the Surber net is usually too large to be used in springs (see Gerecke *et al.* 2007), and the Bou-Rouch pump and the core sampler only collect sediment and interstitial samples.

Our results showed that macroinvertebrate community structure estimated by traps and washing vegetation can be considered comparable to those obtained with net. The four taxa exclusively collected by the net, Hydropsychidae, Limnephilidae, Tipulidae, and Hydrophilidae, have body sizes larger than the opening of the traps (Tachet *et al.* 2000). Furthermore Hydropsychidae, Limnephilidae, and Tipulidae rarely inhabit aquatic vegetation, and Hydrophilidae organisms are very mobile and could escape during vegetation collection (Tachet *et al.* 2000). Although similar communities were collected by both net sampling and combined vegetation washing and trap sampling, there were some differences. The two methods produced different results for Diptera and Coleoptera which was probably related to

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issue with single-habitat protocols. For example, traps and washing vegetation probably underestimated the presence of taxa not associated with vegetation or not attracted by meat. The abundances of organisms collected by traps and vegetation washing were significantly lower than those collected by net. Therefore the impact of these protocols on spring fauna would be expected to be lower, at least on some taxa. In addition, net sampling requires brushing, scraping, digging, and squeezing of different microhabitats and substrata, which cause disturbance of springs habitats and unknown recovering times. The use of traps is more time-consuming than other methods, because they require an additional visit to the springs to be removed. Finally, some sampling methods cannot be suitable in peculiar habitat morphologies: for example, some springs lack any kind of vegetation, and traps cannot be placed in hygropetric springs, where the sediment layer is too thin, or in helocrene springs, that often are too deep. Since spring fauna shows an evident habitat-preferences (von Fumetti *et al*. 2006), single micro-habitat protocols should be used only to survey specific target taxa or habitats. As a consequence, a multi-habitat methodology allows a better estimation of the overall biodiversity. In order to obtain more comparable results, Gerecke *et al*. (2007) recommended sampling available habitats, using proportional sampling time for each substratum and including transitional zones among different substrata since they may host specialised taxa. In addition, the multi-habitat proportional sampling is considered by the Water Framework Directive (Directive 2000/60/EC) as the best approach for assessing macroinvertebrate diversity.

Analysed methods could be improved in order to be more effective. Traps may benefit from a bigger opening for the collection of organisms with larger body size and vegetation-washing method could be applied on more replicas of vegetation samples. Furthermore, surface sediment samples could be added to the combined method of traps and washing vegetation.

In conclusion, the net and the vegetation washing with traps show different features and

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effectiveness, even though both protocols give very similar qualitative results. Indeed, the use of the net, with a multi-habitat proportional approach, provides more accurate and complete information, but also significant impacts on the biotic and abiotic components of springs. For these reasons, this method is only recommended for biodiversity inventories. On the other hand, traps and vegetation washing are still reliable methods with less negative effects on springs ecosystems, thus they are more suitable for ecological studies focused on the analysis of the community structure.

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**Formattato:** Corpo testo, Rientro: Sinistro: 0 cm, Sporgente 0,5 cm, Interlinea: doppia

**Eliminato:** ¶

Table 1: Spring sampling methods and relative references.

| Methods | References |
|---|---|
| **Surber sampler** | Erman & Erman 1995<br>Erman 1998<br>Zollhöfer 1999<br>Smith et al. 2003<br>Von Fumetti et al. 2006<br>Barquín, & Death 2008<br>Gerecke et al. 2011 |
| **Hand or kick net** | Williams 1991<br>Gerecke & Cantonati 1998<br>Hahn HJ. 2000<br>Myers & Resh 2002<br>Mezzanotte & Sambugar 2004<br>Mori & Brancelj 2006<br>Lencioni 2007<br>Staudacher & Füreder 2007<br>Ilmonen et al. 2012<br>Kubíková et al. 2012<br>Martin & Brunke 2012<br>Rader et al. 2012<br>Spitale 2012<br>Tichá et al. 2012 |
| **Collection at sight** | Williams 1991<br>Bonettini & Cantonati, 1998;<br>Gerecke & Cantonati 1998<br>Myers & Resh 2002<br>Gerecke & Di Sabatino 2007 |
| **Bou Rouch pump** | Crema et al. 1996 |
| **Sweep net** | Crema et al. 1996<br>Sambugar et al. 2006 |
| **Core sampler/ sediment sample** | Gooch et al. 1991<br>Myers & Resh 2002<br>Dumnicka et al. 2007<br>Staudacher & Füreder L. 2007<br>Worthington Wilmer et al. 2008<br>Takhteev et al. 2010;<br>Koperski et al. 2011<br>Spitale 2012 |
| **Traps** | Bottazzi et al. 2011 |
| **Drift tube or net** | Stoch et al. 2008<br>Bottazzi 2010 |
| **Squeezing mosses or washing vegetation** | Bottazzi et al. 2011<br>Gerecke et al. 2011<br>Spitale 2012 |
| **Emergence traps** | Erman & Erman 1995<br>Erman 1998<br>Gathmann & Williams 2009 |

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643 Table 2: List of taxa with related methods of collection.
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| Taxa | Net | Traps | Vegetation washing |
|---|---|---|---|
| Leuctridae | + | + | + |
| Lepidostomatidae | + | + | + |
| Philopotamidae | + | + | + |
| Sericostomatidae | + | + | + |
| Chironomidae | + | + | + |
| Stratiomyidae | + | + | + |
| Psychodidae | + | + | + |
| Limoniidae | + | + | + |
| Dixidae | + | + | + |
| Scirtidae | + | + | + |
| Hydraenidae | + | + | + |
| Niphargidae | + | + | + |
| Gasteropoda | + | + | + |
| Collembola | + | + | + |
| Veliidae | + | + | |
| Ceratopogonidae | + | + | |
| Haliplidae | + | + | |
| Dytiscidae (adults and larva) | + | + | |
| Hirudinea | + | + | |
| Hydrachnidiae | + | + | |
| Nemouridae | + | | + |
| Heptageniidae | + | | + |
| Polycentropodidae | + | | + |
| Beraeidae | + | | + |
| Ptychopteridae | + | | + |
| Simuliidae | + | | + |
| Odonata | + | | + |
| Hydropsychidae | + | | |
| Limnephilidae | + | | |
| Hydrophilidae | + | | |
| Tipulidae | + | | |
| Empididae | | + | |

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**Figure captions**

Figure 1: Logarithm of taxa abundances for the three distinct methods. Tested by ANOVA, differences in abundances between methods resulted significant ($F = 16.180$; $p < 0.001$).

Figure 2: Comparison of taxa abundances (log transformed) using net sampling and combing traps and vegetation washing. Tested by ANOVA, differences in abundances between methods resulted significant ($F = 9.464$; $p = 0.012$).

Figure 3: NMDS ordination of the three distinct methods (stress = 0.15). Black points are the centroids of methods (veg = vegetation washing). Grey points are sampled communities.

Figure 4: NMDS ordination of the net and the traps plus vegetation washing (stress = 0.13). Black points are the centroids of methods (traps+veg = traps plus vegetation washing). Grey points are sampled communities.

Figure 5: NMDS ordination of the net and the traps+vegetation washing for Trichoptera, Coleoptera, and Diptera. Black points are the centroids of methods (traps+veg = traps plus vegetation washing). Grey points are sampled communities.